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Clinical Significance of Hepatic HCV RNA in Patients With Chronic Hepatitis C Demonstrating Long-Term Sustained Response to Interferon-Alpha Therapy

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Whether sustained biochemical response and absence of serum HCV RNA in the 6–12 months following suspension of interferon-α (IFN-α) therapy reflect definitive viral clearance in patients with chronic hepatitis C virus (HCV) infection is controversial. To obtain more information on this topic, HCV RNA was sought in both liver and serum samples of 25 long-term responders who were followed for a median period of 39 months (range 21–79) after discontinuation of IFN-α. Liver biopsy was undertaken before and 6 to 12 months after IFN-α withdrawal. Liver and serum HCV RNA were tested by a nested polymerase chain reaction. Twenty-two patients (88%) tested negative for both liver and serum HCV RNA, two patients had detectable HCV RNA in both liver and serum, and one patient showed persistent HCV RNA only in the liver. Post-treatment liver histology improved markedly in all patients, including those with viral persistence. During further follow-up, biochemical remission was maintained in all patients except one in whom both serum and liver specimens remained HCV RNA positive. The data indicate that the large majority of long-term responders test negative for HCV RNA in the liver, which suggests definitive eradication of HCV RNA infection. ***J. Med. Virol. 55:7–11, 1998.***

KEY WORDS: hepatitis C virus; liver HCV RNA; interferon-α, therapy; long-term responders

INTRODUCTION

Interferon alpha (IFN-α) is currently the only drug licensed for the treatment of chronic hepatitis C virus (HCV) infection. However, only about 10–25% of patients treated with IFN-α show a long-term sustained response, defined as persistent normalization of serum alanine aminotransferase (ALT) concentration lasting at least 6 months after the discontinuation of treatment [Fried and Hoofnagle, 1995; Terrault and Wright, 1995]. Moreover, whether sustained biochemical improvement in these patients reflects viral clearance is still controversial [Lau et al., 1993]. Some investigators have suggested that IFN-α therapy leads to a complete eradication of HCV in long-term responders [Shindo et al., 1992; Reichard et al., 1994; Romeo et al., 1994], whereas others have reported persistence of HCV viremia in a subset of long-term responders [Kakumo et al., 1993; Marcellin et al., 1994], even in those with improved liver histology [Saracco et al., 1994]. In addition, it is also debated whether absence of detectable HCV RNA in serum 1 year after IFN-α discontinuation indicates definitive eradication of HCV infection [Chemello et al., 1996; Vento et al., 1996]. Additional indices are needed to identify the patients in whom relapse of the disease may be confidently excluded and to define better the natural history of long-term responders. This is relevant also in view of a recent report on the development of hepatocellular carcinoma in a long-term responder with viremia [Shindo and Okuno, 1996].

HCV RNA determination in liver tissue performed at the end of IFN-α therapy has been shown to predict the sustained normalization of serum ALT for 6–12 months [Shindo et al., 1995], and the disappearance of hepatic HCV RNA has been suggested to be a more reliable

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indicator of disease remission than clearance of serum HCV RNA [Shindo et al., 1995]. Unfortunately, because of the short follow-up of these studies, no definitive conclusions can be drawn on whether liver HCV RNA determinations predict long-term outcome in long-term responders.

In a previous pilot study, it was found that viremia persisted at very low titres and with a fluctuating pattern in about 38% of long-term responders [Bruno et al., 1997], but the clinical significance of such a virologic pattern remained unclear. We therefore investigated the relation between presence of HCV RNA in serum and the liver and their value in predicting the long-term outcome in patients with sustained biochemical response to IFN-α treatment.

PATIENTS AND METHODS

Patients and Monitoring Procedures

From January 1990 to December 1993, 204 consecutive patients with chronic hepatitis C attending our centre were treated with IFN-α therapy according to different treatment schedules in the context of two randomized controlled trials (maximum dosage, 9 MU three times a week for 18 months; minimum dosage, 3 MU three times a week for 16 weeks). Thirty-eight patients (19%) showed a long-term sustained response, defined as normal ALT levels at monthly determinations for at least 6 months after discontinuation of treatment. Twenty-five of these 38 patients, whose posttreatment frozen liver specimens were available for HCV RNA detetermination, were considered for this study.

Starting 6 months after IFN-α discontinuation, serum liver enzymes were monitored in the 25 patients at 3-month intervals for an additional 15 to 73 months (median follow-up after therapy, 39 months). Biochemical relapse was defined as an elevation of serum ALT above the upper limit of the normal range at two consecutive determinations.

Liver biopsy was obtained from all patients before treatment and was repeated 6 to 12 months after the discontinuation of therapy, according to the study designs and in agreement with generally accepted criteria for evaluation of histological outcome [Craxì et al., 1996a]. A third liver biopsy was also undertaken in six patients after 44 to 68 months of follow-up (median, 50 months). About half of the liver specimen was fixed in formalin for histological examination. A second portion of liver tissue weighing approximately 30 mg was snap frozen in liquid nitrogen immediately after being obtained. It was stored at −80°C until extraction and assay for HCV RNA.

Serum samples collected before and after treatment, at the time of liver biopsies and at the end of follow-up, were tested for HCV RNA. Serum for HCV RNA was collected within 1 hour of blood sampling and immediately frozen at −80°C until testing.

Histologic diagnosis was made using both hematoxylin and eosin and reticulin-stained liver sections according to a recent international scoring system for chronic hepatitis [Desmet et al., 1994]. Samples were read by one pathologist who was blinded to clinical data and to the sequence in which biopsy specimens had been obtained.

HCV RNA Detection and Genotyping

Frozen liver biopsy samples were weighed, and homogenized rapidly in 100–200 μl of an 8-M guanidine HCl solution and then an additional volume of homogenization solution was added to give 500 μL per 25 mg of tissue. Serum was separated from red blood cells within 1 hour of phlebotomy and frozen at −80°C to avoid prolonged exposure of virus to the clot. RNA was extracted from 100 μL of liver homogenate or 100 μL of serum by the guanidinium isothiocyanate-phenol-chloroform method and amplified by a reverse transcription polymerase chain reaction (RT-PCR) using nested primers from the 5′ noncoding region [Ribero et al., 1993].

All RT-PCR assay runs included both low positive and negative controls for serum or liver tissue obtained from patients with drug-induced hepatitis, negative for all viral hepatitis markers. Comparison with serially diluted serum or RNA extracted from liver homogenate samples by the Quantiplex HCV RNA branched DNA 2.0 assay (Chiron Corporation, Emeryville, CA) for genotypes 1, 2, and 3 indicated that this nested PCR assay was able to detect reliably 100 HCV RNA copies per mL of serum or per g of liver tissue for each genotype.

HCV genotypes were identified by RT-PCR amplification with nested type-specific primers from HCV core and NS5 regions [Donato et al., 1997]. For the identification of genotypes 1a, 1b, 2, and 3a newly designed type-specific primers were used in the second round of PCR:

1. Core region: 1aF: 5′- GAG CCA TCC TGC CCA CCC CA -3′, nt 507 to 527 for subtype 1a; 2abF: 5′- ACC GGC AAG TCC TGG GGA A -3′, nt 555 to 573 for type 2; VF: 5′- CAA AGC GCG TCG GAG CGA AGA CC -3′, nt 539 to 561 for subtype 3a.
2. NS5b region: POL 8710: 5′- TGT CGG TCG CGC ACG ATG CAT -3′, nt 8710 to 8730 and C.2: 5′- ACT TCT GGC CCG ATG TCT CCA GA -3′, nt 9119 to 9097 for subtype 1b; POL 9059: 5′- AGC CAC CCG TGT CAG TTC GTG G -3′, nt 9059 to 9038 for type 2; POL 8653: 5′- GAG ATG CTC CAC AGG CCA CT -3′, nt 8653 to 8672 for subtype 3a.

Only consistent results from both core and NS5b regions were considered for the final assignment of HCV genotype.

Statistical Analysis

Data are reported as mean and standard errors of the mean or medians. Changes of histological variables over time were assessed as the difference between last and initial values. Comparisons between groups were made using the Mann-Whitney test and the chi-square or Fisher's exact tests. Pairwise comparisons between

TABLE I. Characteristics of Patients With Chronic Hepatitis C Before Treatment With Interferon-α in the Context of Two Randomized Controlled Trials

	Treated patients (n = 204)	Patients showing long-term response (n = 38)	Long-term responders with available liver HCV RNA (n = 25)
Age (years, mean ± SEM)	47 ± 1	48 ± 2	48 ± 3
Male (%)	119 (58)	21 (55)	14 (56)
No. with cirrhosis (%)	42 (21)	4 (11)	3 (12)
ALT[a] (IU/L, mean ± SEM)	161 ± 7	166 ± 18	165 ± 19

[a]Normal values <45 IU/L.

data at entry and those obtained at the end of the observation were performed using the Wilcoxon test. All analyses were two-sided, with a significance level of 0.05.

RESULTS

The 25 patients whose liver tissue was available for HCV RNA determination had similar demographic, clinical, and histological features compared with the 38 long-term responders that we have observed in our centre (Table I). The individual characteristics of the former are shown in Table II. There were 14 men and 11 women with a mean age of 48 years (range, 18 to 69). Risk factors for viral infection were previous drug addiction in two patients, blood transfusion in five, and unknown in 18. Serum HCV RNA was positive in all 25 patients before therapy. Genotype 1b was detected in six of them, genotypes 2 and 3a in 12 and four, respectively, and the genotype could not be classified in three.

Six to 12 months after discontinuation of therapy, HCV RNA was no longer detectable in liver and serum samples of 22 (88%) of the 25 patients. Two patients (nos. 18 and 24) had detectable HCV RNA in both liver and serum, and in a third (no. 23) HCV RNA persisted in the liver but not in serum (Table II). The three patients who remained positive for liver HCV RNA had genotype 2, were older than those who became negative for HCV RNA (65 vs. 49 years), and two of them had cirrhosis. Neither the dose or duration of IFN-α administration nor HCV genotype were associated with persistence of liver HCV RNA, with or without viremia.

Results of serum HCV RNA determination at the end of follow-up (median, 39 months; range 21 to 79 months) did not differ from those observed at the time of post-treatment liver biopsy (Table II).

Patients showing persistent liver HCV RNA had a significantly higher mean histological activity score at baseline, reflecting more severe fibrosis ($P < 0.05$) and lobular necrosis ($P < 0.05$). After IFN-α administration, liver histology improved in all patients ($P < 0.05$).

Six patients (nos. 1, 8, 9, 13, 16, and 24) underwent a third liver biopsy 44 to 68 months after IFN-α withdrawal. No changes in liver HCV RNA status were found, and a slight further improvement of histology was observed.

During follow-up, all the 22 patients testing negative for liver HCV RNA maintained normal ALT levels, whereas biochemical relapse occurred in one (no. 18) of the three patients with detectable HCV RNA in the liver 21 months after IFN-α discontinuation.

DISCUSSION

The present study was aimed at characterizing clinical and virological features and long-term outcome of patients with chronic HCV infection showing a sustained response to IFN-α treatment. To our knowledge, this study covers the longest follow-up period of a meaningful number of long-term responders who underwent liver HCV RNA determination after discontinuation of IFN-α.

Twenty-two of the 25 long-term responders had mild to moderate chronic liver disease at baseline. This is in agreement with findings from other studies indicating that histological expression of liver disease is one of the most important host factors affecting the response to IFN-α therapy [Matsumoto et al., 1994; Sharara et al., 1996].

HCV infection was eradicated in the large majority of patients in the present series. After stopping treatment, HCV RNA persisted in the liver in only three long-term responders (12%). Although highly variable rates of viral eradication have been reported in long-term responders [Fried and Hoofnagle, 1995], the present results concur with those of Shindo et al. [1995] in showing persistent liver HCV RNA in 10% of long-term responders.

A good correlation between tissue and serum HCV RNA test results was found, except in one patient in whom HCV RNA was detected in the liver but not in serum. Such a discrepancy has also been reported by others [Shindo et al., 1995; Reichard et al., 1995] and may be related to the higher concentration of HCV RNA in liver tissue as compared with serum [Nakagawa et al., 1994; Idrovo et al., 1996] as the liver is the main site of viral replication. This leads to a higher sensitivity of liver compared to serum HCV RNA tests, a difference which may be more marked following IFN-α treatment when the viral load decreases [Shindo et al., 1991]. Four years after discontinuation of IFN-α, our patient with a positive liver HCV RNA test but a negative serum one maintained a complete biochemical response, with persistently HCV RNA negative serum. This was not the case in another patient described by Reichard et al. [1995], who developed biochemical and virological relapse during follow-up.

On the other hand, the specificity of HCV RNA tests is a matter of debate [Zaaijer et al., 1993]. Of the 22 patients without liver HCV RNA included in the present study, three (nos. 4, 13, and 16) had been previously enrolled in a pilot study involving serial plasma HCV RNA determinations over 24 months following IFN-α discontinuation (Bruno et al., 1997). They had intermittently HCV RNA at a very low titre.

TABLE II.[a]

Patient no.	Sex	Age (yr)	Genotype	ALT[b] (IU/L)	Liver histology[b]	Follow-up[c] (mos.)	Liver HCV RNA[d]	Serum HCV RNA[d]
1	F	28	3a	354	CH mild	47	—	—
2	F	51	1b	56	CH mild	50	—	—
3	F	35	1b	177	CH mild	30	—	—
4	F	52	2	203	CH mild	76	—	—
5	F	50	1b	78	CH mild	31	—	—
6	F	48	1b	185	CH mild	30	—	—
7	F	60	2	391	CH mild	39	—	—
8	F	53	2	293	CH mild	47	—	—
9	M	60	2	96	CH mild	54	—	—
10	M	31	3a	111	CH mild	21	—	—
11	F	34	2	106	CH mild	38	—	—
12	M	27	3a	113	CH mild	24	—	—
13	M	56	UC	351	CH moderate	74	—	—
14	M	62	2	58	CH moderate	53	—	—
15	M	48	1b	133	CH moderate	53	—	—
16	M	59	1b	144	CH moderate	53	—	—
17	F	51	3a	77	CH moderate	36	—	—
18	M	69	2	105	CH moderate	30	+	+
19	M	36	2	126	CH moderate	38	—	—
20	M	68	UC	114	CH moderate	79	—	—
21	F	30	2	207	CH moderate	33	—	—
22	M	18	UC	247	CH moderate	29	—	—
23	M	58	2	156	Cirrhosis	49	+	—
24	M	69	2	165	Cirrhosis	70	+	+
25	M	59	2	78	Cirrhosis	30	—	—

[a]CH, chronic hepatitis; UC, unclassified type. Normal values for ALT < 45 IU/L.
[b]Baseline evaluation.
[c]From treatment discontinuation.
[d]Evaluated 6 to 12 months after stopping interferon therapy.

During a follow-up period now extended to 76, 74, and 53 months, respectively, the response persisted in all three patients and a third liver biopsy was repeated in two of them (nos.13 and 16), which confirmed the negativity of liver HCV RNA. In view of its higher sensitivity [Guido and Thung, 1996], repeatedly negative liver HCV RNA tests strongly indicate viral eradication; and we suggest that inconsistent low-titre positivity of plasma HCV RNA determinations, especially when observed early during the posttreatment follow-up, should raise doubts regarding their specificity.

Results from a recent study suggest that the response to IFN-α treatment is permanent when serum HCV RNA tests are negative 1 year after therapy withdrawal, whereas long-term responders who remain viremic are at risk of hepatitis reactivation [Chemello et al., 1996]. By contrast, another group has reported that late relapse occurs in almost all long-term responders despite undetectable serum HCV RNA 1 year after discontinuation of treatment [Vento et al., 1996]. The present data, also on liver tissue, are in agreement with Chemello et al.'s [1996] findings as so far no patient with negative liver and serum HCV RNA tests has developed a late relapse.

Interestingly, two of the three long-term responders who did not clear hepatic HCV RNA had liver cirrhosis. A long-term biochemical response to IFN-α treatment in the absence of viral clearance was previously reported to be more frequent in patients with cirrhosis [Craxì et al., 1996b]. Treatment with IFN-α may select escape mutants [Weiner et al., 1992; Martell et al., 1992] and lack of viral clearance in patients with more advanced liver disease might reflect the higher degree of diversity of HCV quasispecies [Honda et al., 1994].

Histological indexes of necroinflammatory activity improved after treatment with IFN-α, and this was not confined to patients who achieved viral clearance. In the six patients who underwent a third liver biopsy, a further improvement of liver histology was observed, thus suggesting that a long period of time is needed for remission of the necroinflammatory damage to be fully manifest. However, this issue is still controversial [Saracco et al., 1994; Chemello et al., 1996], and larger patient populations are needed to evaluate the relationship between viral clearance and histological improvement in long-term responders.

In conclusion, negative liver HCV RNA tests are associated with persistent biochemical and virological response to IFN-α. In long-term responders who do not achieve viral clearance despite the favourable histological changes induced by IFN-α treatment, late relapses of disease may occur. Such patients should be followed carefully in view of the recent report of the development of hepatocellular carcinoma in a long-term responder with viremia [Shindo and Okuno, 1996].

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